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Association between *Bifidobacteriaceae* and the clinical severity of root caries lesions

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Background/aims: The isolation of members of the family *Bifidobacteriaceae* (bifids) from oral samples has been sporadic and a recent cloning study has suggested that they are not detectable in root caries lesions.
Methods: To better understand the presence of bifids in root caries we obtained clinical samples (15 of each) from sound exposed root surfaces, leathery remineralizing root lesions, and soft active root lesions. We investigated each for the presence of bifids using a mupirocin-containing selective medium and identified the isolates using 16S recombinant RNA sequencing.
Results: The proportion of bifids, as a percentage of the total anaerobic count, was significantly related to the clinical status of the sites sampled, being 7.88 ± 1.93 in the infected dentine from soft lesions, 1.61 ± 0.91 in leathery lesions, and 0.05 ± 0.39 in plaque from sound exposed root surfaces. Bifids were isolated from all soft lesions, 13 of 15 leathery lesions, and five of the plaque samples. *Bifidobacterium dentium* was isolated from four of the plaque samples, from 13 samples from leathery lesions, and from 12 of the 15 samples of infected dentine from the soft active lesions. *Parascardovia denticolens* and *Scardovia* genomospecies C1 were each isolated from samples associated with all three clinical conditions whereas *Scardovia inopicata* and *Bifidobacterium subtile* were both isolated from the infected dentine of the leathery and soft lesions. *Bifidobacterium breve* was isolated from the infected dentine of soft root caries lesions.
Conclusion: Bifids may be routinely isolated from root caries lesions using appropriate cultural methods.

Key words: *Bifidobacterium;* dental caries; fructose-6-phosphate phosphoketolase test; polymerase chain reaction; 16S rRNA

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Bifidobacteriaceae (bifids) are acidogenic and aciduric, gram-positive, pleomorphically branched, non-motile, non-spore-forming, and non-filamentous rods that can be grouped on the basis of one of six different ecological niches that they occupy: the human intestine, oral cavity, food, the animal gastrointestinal tract, the insect intestine, and sewage (37). Around 32 species of bifids have been described but the range of bifids reported to be commensal organisms in the oral cavity is apparently restricted to *Bifidobacterium dentium*, *Scardovia inopicata*, and *Parascardovia denticolens*, which have been isolated from carious dentine and dental plaque. The latter two genera were differentiated from the genus *Bifidobacterium* on the basis of their G + C mol%, 16S recombinant RNA (rRNA), and heat-shock protein 60 gene sequence analysis (21). More recently, *Alloscardovia omnicolens*, (20) isolated from human infections, was described as a new closely related genus while '*Scardovia* genomospecies C1' (28) was reported from carious dentine and '*Scardovia* sp. T01-04' (18) was identified, on the basis of 16S rRNA sequencing, from cellular samples taken from oral cancer cells.

There have been only sporadic reports of bifids being isolated, or identified in 16S rRNA cloning and sequencing studies, from the mouth usually in conjunction with caries (3, 9, 11, 19, 23, 26, 27, 32, 35) and not from the healthy mouth (1). In a survey of oral bifids in carious dentine and in supragingival dental plaque only *B. dentium*, *S. inopicata*, and *P. denticolens* were isolated (26) but previously Hoshino (19) isolated bifids from two of four dentinal lesions and 11 of 19 isolates were identified as '*Bifidobacterium breve*-like' and the remainder as *B. dentium*. In another Japanese study (17) *Bifidobacterium adolescentis* was isolated from the saliva of four of 10 young adults and *Bifidobacterium urinalis* and *Bifidobacterium longum* were each isolated from the saliva of one of the 10 subjects investigated (17) while *B. longum* and *B. breve* were reported from infected root canals (8). *Bifidobacterium* spp., isolated from advanced root caries lesions (34), were found to be capable of acidogenesis and to produce a final pH in glucose-containing broth below 4.2 (34) sufficient to cause extensive demineralization of tooth tissues. More recently, in studies identifying the presence of individual taxa on the basis of homology between sample DNA and probes, high levels of bifids have been associated with childhood dental caries and it was hypothesized that they may play a large role in caries pathogenesis (3). However, using a cloning methodology, the microbiota of root caries lesions in 11 subjects was dominated by *Actinomyces* spp., lactobacilli, *Streptococcus mutans*, *Enterococcus faecalis*, *Selenomonas* sp. clone CS002, *Atopobium* and *Olsenella* spp., *Prevotella multisaccharivorax*, *Pseudoramibacter alactolyticus*, and *Propionibacterium* sp. strain FMA5 and bifids were not reported (30). It may also be that bifids, unlike other caries-associated organisms including mutans streptococci, lactobacilli and yeasts, are only sporadically isolated in cultural studies because of the lack of suitable selective isolation media. Nonetheless there is an apparent discrepancy between the molecular data and the data derived from cultural studies, which suggest that bifids should be part of the microbiota present in infected dentine associated with caries lesions.

To try to understand the discrepancy between the molecular studies of the microbiota of occlusal and root caries lesions we have employed a modification of the novel selective medium (MTPY) previously used to isolate non-glucose fermenting bifids from the cecum of chickens (31). We have isolated and identified bifids from soft, active root carious lesions, leathery, remineralizing root carious lesions, and from supragingival plaque recovered from sound surfaces of exposed roots. The proportion of bifids in the samples was related to the clinical severity of lesions.

Materials and methods

Clinical status of subjects

Patients selected for the study were adults, treated in the Prosthodontics Department of the King's College London Dental Institute. The patients' age ranged from 30 to 85 years and there were 33 males and 11 females. The patients were fully or partially dentate, with or without dentures. They were medically stable with no active, acute medical conditions and not receiving any antibiotic treatment. The procedure was fully explained to the patient and verbal consent was obtained. In the case of the patient having treatment under sedation, a written consent was obtained before any treatment. Ethical approval for collection of the root caries and sound exposed root surface samples was obtained by the Guy's Hospital Research Ethics Committee.

Sampling sites

Samples were obtained from the supragingival plaque of sound exposed root surfaces (n = 15), from the infected dentine of soft, active root caries lesions (n = 15) and from the infected dentine of leathery root caries lesions (n = 15). The involved tooth, site of the lesion, and lesion color, dimensions, and distance from the gingival margin were recorded (5, 16). Before obtaining the samples, the superficial plaque was brushed away from the lesion site using a new toothbrush and water; the patients rinsed with tap water. The samples were taken by running a sterile excavator vertically through the lesion (5) or the sound exposed root surface (plaque sample) and immediately suspending each in 1.0 ml of anaerobically pre-reduced Chalgren–Wilkins broth (Oxoid, Basingstoke, UK). The samples were placed on ice and processed within 1–4 h.

Sample processing

The clinical samples were dispersed by thoroughly vortexing for 10 s with sterile glass beads and one in 10 dilutions were prepared in Chalgren–Wilkins broth. Aliquots of 100 µl of appropriate dilutions were spread on MTPY medium (mupirocin-containing selective medium) for the isolation of bifids, which consisted of proteose peptone (Oxoid, Basingstoke, UK) 7 g, soya peptone (Oxoid) 5 g, yeast extract (Oxoid) 5 g, glucose 15 g, raffinose 5 g, cysteine.HCl 0.5 g, K_2HPO 2 g, $MgCl_2.6H_2O$ 0.5 g, $ZnSO_4.7H_2O$ 0.25 g, $CaCl_2$ 0.15 g, $FeCl_3$ 0.001 g, agar 15 g and Tween-80 1 ml/l deionized water. The medium was autoclaved at 15 psi for 15 min, cooled and 1 ml glacial acetic acid and 1 ml of a 5 mg/ml mupirocin 50% ethanolic solution were added, and plates were poured. The pH of the MTPY medium was approximately 4.9. Rogosa agar medium (Oxoid) was used for the isolation of lactobacilli, bacitracin mitis salivarius agar (BMSA) (13) for the isolation of mutans streptococci and Sabouraud dextrose agar (SAB; Oxoid) for the isolation of yeasts. The SAB plates were incubated aerobically at 37°C for 3 days, the MTPY, BMSA, and Rogosa plates were incubated anaerobically for 4 days at 37°C. The number of total cultivable bacteria was determined by plating dilutions onto fastidious anaerobe agar (FAA, LabM, Bury, UK) supplemented with 5% (volume/volume) defibrinated horse blood and the inoculated FAA plates were incubated anaerobically at 37°C for 7 days.

Enumeration of organisms in samples

The total number of cultivable bacteria in the samples was determined by enumerating the number of colonies on the FAA plates. The numbers of mutans streptococci, lactobacilli, and yeasts in the samples were determined as previously described (5). To determine the numbers of bifids in each sample the total number of presumptive bifid colonies on the MTPY was counted and the number of each colony type was determined. The presumptive bifids were gram-stained and those with characteristic gram-positive, bifid-shaped, or pleomorphic rods were subcultured. Typically 25–30 of each colony type were subcultured from each sample and tested for fructose-6-phosphate phosphoketolase (F6PPK) activity according to the method described by Orban and Patterson (29) in which cells are lyzed by the addition of cetyltrimethylammonium bromide. DNA was also extracted from all presumptive colonies by suspending cells in 50 µl Tris–ethylenediaminetetraacetic acid (TE)buffer and 0.5% Tween-80 solution to which 1 µl of a 10 mg/ml Proteinase K solution was added and the cells were heated for 2 h at 55°C and for a further 5 min at 95°C. The extracts were cooled by placing on ice and stored at

−20°C. For immediate use, a suspension was prepared in 100 µl double-distilled water and used as the DNA template for the polymerase chain reactions (PCRs).

A partial sequence of the 16S rRNA gene was obtained by first amplifying the gene fragment using the genus-specific PCR primers Bif164mod 5′-GGGTG GTAATRCCSRATG-3′ and Bif662mod 5′-CCACCGTTACACCGRGAA-3′ modified from those previously reported by Venema and Maathuis in 2003 (36). On some occasions it was necessary to use universal primers (24) 63F and 1387F to amplify the 16S rRNA gene and to sequence the amplicons using the 16F primer. PCR amplification was achieved using the following reaction mixture (25 µl final volume) which comprised of 4 µl $MgCl_2$ 25 mM, 2.5 µl 10 × buffer, 1 µl dNTPs (3.125 mM), 1 µl Bif164mod forward primer, 1 µl Bif662mod reverse primer, 0.5 µl *Taq* DNA polymerase (ABGene, Epsom, UK), 14 µl distilled H_2O, and 1 µl DNA extract. The thermal cycling conditions were an initial denaturation at 94°C for 5 min, denaturation at 94°C for 60 s, annealing at 58°C for 30 s, extension at 72°C for 90 s, repeated for 34 cycles and final extension at 72°C for 7 min.

PCR products were cleaned by polyethyleneglycol precipitation and sequenced in reactions containing 2 µl PCR product, 0.5 µl of BigDye v3.0 (Applied Biosystems, Warrington, UK), 1.75 µl of 5 × solution buffer (Applied Biosystems, Warrington, UK), 1.75 µl of sterile ultra high quality (UHQ) water and 4 µl primer (3 pmol). Both strands for each gene fragment were sequenced. The cycling protocol and cleaning of sequencing reaction products were as described in the manufacturer's protocols and sequencing was performed using an ABI 3730xl DNA Analyzer (Applied Biosystems).

Identification of bifids

The 16S rRNA sequence data were manually corrected and edited using BioEdit (15). The sequences were identified using the Ribosomal Database Project (10) or by BLAST searching (2). Phylogenetic trees were constructed using the MEGA 4.0 program (33).

Statistical analysis

To compare the frequency of isolation and proportional representation of individual taxa, data were analyzed using appropriate non-parametric statistical tests within SPSS PC.

Results

The soft, leathery lesions had a similar mean vertical length (3.8 mm) but the soft lesions were wider (4.2 mm) than the leathery lesions (3.7 mm). The mean distance of the soft, active lesions from the gingival margins was 1.0 mm, which was less than the mean distance of the leathery lesions (1.5 mm). Of the soft carious lesions, 47.1% were recorded as light brown, 35.3% were recorded as dark brown, and 17.6% as yellow while 60% of the leathery lesions were dark brown, 26.6% light brown, 6.7% yellow, and 6.7% black.

The frequency of isolation of bifids from the soft active lesions and from leathery root lesions was significantly greater then the frequency of isolation from the sound exposed root surfaces ($\chi^2 = 12.15$; $P < 0.001$ and $\chi^2 = 6.81$; $P = 0.0029$). The number of bifids per sample (as $\log_{10}$[colony-forming units per sample + 1]) was significantly correlated with the number of lactobacilli and yeasts but not with the number of mutans streptococci ($r = 0.398$; $P = 0.005$ and $r = 0.289$; $P = 0.044$, respectively controlling for total number of organisms in the sample) while the proportion of bifids in the samples was significantly correlated with the proportions of lactobacilli and mutans streptococci ($r = 0.537$; $P < 0.001$ and $r = 0.295$; $P = 0.034$, respectively).

The proportion of bifids, as a percentage of the total anaerobic count, was significantly related to the clinical status of the sites sampled. The lowest proportion of bifids was recovered from the supragingival plaque from the sound exposed root surfaces and the highest proportion was recovered from the infected dentine of the soft, active root lesions while the proportion of bifids in the infected dentine of the leathery, remineralizing root lesions was intermediate between these two extreme values (Table 1). The proportions of lactobacilli followed the same pattern and the proportions of mutans streptococci and yeasts were significantly greater in the dentine samples from the soft lesions than from the plaque from the sound root surfaces but not greater than the proportion in the dentine from the leathery lesions.

From the MTPY plates a total of 596 presumptive bifids was identified using 16S rRNA sequencing. We found a small number of isolates that failed to give a positive F6PPK test but were nonetheless identified as bifids, this was most notable for the colonies identified as *Bifidobacterium subtile*. Isolates were positively identified if the partial 16S sequences exhibited ≥ 98% sequence identity with the sequences of type strain and reference strains. Of the isolates identified, 488 were *B. dentium*, 24 were *B. subtile*, 44 were *P. denticolens*, 27 were *S. inopicata*, eight were *Scardovia* genomosp. C1, four were *B. breve,* and one was identified as *B. longum* (Table 2). From the samples yielding bifids, *B. dentium* was isolated from four of five (80%) root surface plaque samples, from all 13 (100%) samples of infected dentine from the leathery lesions and from 12 of the 15 (80%) samples of infected dentine from the soft active lesions. *P. denticolens* and *Scardovia* genomosp. C1 were each isolated from samples associated with all three clinical conditions while *S. inopicata* was isolated from the infected dentine of the leathery and soft lesions. *B. subtile* was isolated from the infected dentine of leathery and soft lesions from the same patient. *B. breve* was isolated from the infected dentine of two of the soft root caries lesions while a very low level of *B. longum* was found in one of the plaque samples from the sound exposed root surfaces. The proportions of *B. dentium* in the infected dentine of soft and leathery root caries lesions were not significantly different but both were

Table 1. Proportional representation, as a percentage of the total anaerobic count (mean ± SE and median) of caries-associated organisms isolated from root lesions and sound root surface samples

	Site sampled		
	Soft, active lesions ($n = 15$)	Leathery, remineralizing lesions ($n = 15$)	Sound exposed root surfaces ($n = 15$)
Mutans streptococci	3.52 ± 1.56 0.67[1]	1.81 ± 0.95 0.08	0.67 ± 0.44 0.009
Lactobacilli	31.18 ± 9.77 7.92[1]	8.97 ± 3.88 0.30[2]	0.13 ± 0.11 < 0.001[3]
Yeasts	0.23 ± 0.13 0.04[1]	0.36 ± 0.34 0.01	2.17 ± 2.13 < 0.001
Bifidobacteriaceae	7.88 ± 1.93 6.38[1]	1.61 ± 0.91 0.10[2]	0.05 ± 0.39 < 0.001[3]

Values with different superscripts in rows are significantly different ($P < 0.05$) from all other values in the row (Friedman and Mann–Whitney tests).

Table 2. Proportional representation of *Bifidobacteriaceae* taxa isolated from individual[5] soft, active and leathery root lesions and sound exposed root surfaces

Sample number	Total bifids	*B. dentium*	*P. denticolens*	*S. inopicata*	*Scardovia* Genomosp. C1	*B. breve*	*B. subtile*
Sound exposed root surfaces[1]							
1	0.04	0.04	–	–	–	–	–
2	0.01	0.002	0.008	–	–	–	–
3	0.14	0.14	–	–	–	–	–
4[4]	0.03	–	0.03	–	–	–	–
5	0.60	0.30	–	–	0.30	–	–
Leathery remineralizing root surface lesions[2]							
1	0.08	0.08	–	–	–	–	–
2	7.24	4.37	2.87	–	–	–	–
3	0.10	0.10	–	–	–	–	–
4	0.27[5]	0.18	0.008	0.06	–	–	0.03
5	0.71	0.71	–	–	–	–	–
6	0.07	0.07	–	–	–	–	–
7	0.42	0.42	–	–	–	–	–
8	0.02	0.02	–	0.002	0.001	–	–
9	0.02	0.02	–	–	–	–	–
10	0.76	0.76	–	–	–	–	–
11	12.50	12.5	–	–	–	–	–
12	1.93	1.93	–	–	–	–	–
13	0.06	0.03	0.03	–	–	–	–
Soft active root caries lesions[3]							
1	11.27	3.19	8.08	–	–	–	–
2	2.05	2.05	–	–	–	–	–
3	0.40[5]	0.34	–	–	–	–	0.06
4	24.88	21.46	–	–	3.42	–	–
5	11.50	11.5	–	–	–	–	–
6	0.46	0.43	–	–	–	0.03	–
7	6.38	–	–	–	6.38	–	–
8	15.45	15.45	–	–	–	–	–
9	0.18	0.18	–	–	–	–	–
10	2.36	–	–	1.2	–	0.36	–
11	10.86	–	10.86	–	–	–	–
12	15.44	15.44	–	–	–	–	–
13	1.17	1.17	–	–	–	–	–
14	0.12	0.12	–	–	–	–	–
15	13.49	13.49	–	–	–	–	–

[1]No bifids were isolated from 10 of 15 sound, exposed root surfaces.
[2]No bifids were isolated from 2 of 15 leathery root caries lesions.
[3]Bifids were isolated from all soft active root carious lesions.
[4]Proportion of *B. longum* in sample 4 was 0.0006% of total anaerobic colony count.
[5]*Bifidobacterium subtile* also isolated from one other soft root carious lesions in this subject. Leathery lesion sample and soft lesion samples from same patient.

significantly greater than the proportion recovered from the plaque from the sound exposed root surfaces. The frequency of isolation and the proportions of bifids, other than *B. dentium*, were not significantly related to the clinical status of the sample sites.

Discussion

Members of the family *Bifidobacteriaceae* have both acidogenic and aciduric characteristics relevant to the progression of dental caries, including root caries (4, 7). They also exhibit a range of abilities to degrade complex carbohydrates, including dextrans (22), which might potentiate the production of demineralizing acids within the oral biofilm in the absence of dietary fermentable carbohydrates. Despite these phenotypic properties the reports of the isolation of bifids from healthy or diseased intraoral sites have generally been sporadic and then usually in relation to active progressing caries (3, 9, 19, 26, 34). They are reported not to be present in the healthy mouth (1). In a recent study (30), using a 16S rRNA cloning and sequencing approach for the examination of the microbiota associated with health and disease, no bifids were identified among the 3544 clones derived from the microflora of 11 root caries lesions and samples of plaque taken from sound root surfaces in the same subjects. In that study, < 100 clones were examined per sample so the detection limit for any taxon was < 1%, assuming no bias in the detection of any individual taxon. However, it is well established that there is bias against the detection of high G + C organisms including members of the phylum *Actinobacterium* which includes *Actinomyces* and *Bifidobacteriaceae* perhaps because of their resistance to cell lysis, primer bias, or the problems encountered by DNA polymerases to amplify DNA sequences containing a high content of G + C (14). A recent comprehensive analysis of 27f primers has also provided clear evidence that commonly used sequences fail to amplify Bifidobacteriales as there are three mispairings between the Bifidobacteriales sequence and the 27f sequence most commonly used (12). These difficulties might explain why bifids were not detected in any one of the 11 active root carious lesions examined using a cloning methodology. As we isolated bifids from all 15 active root carious lesions and from the majority of leathery lesions the cloning study would appear to have used a protocol that selected against the detection of bifids, and perhaps other actinobacteria. The 'true' proportion of bifids among the microbiota of the infected dentine is difficult to determine but it is likely that the proportions reported here may be the maximum proportions because a large proportion of the microbiota present in root carious lesions are unculturable (30), or perhaps cultured but not characterized using 16S rRNA sequencing. The use of more appropriate primers (12) will no doubt enable bifids to be reliably identified among the microbiota of root caries lesions.

We improved the likelihood of detecting bifids in the oral samples examined because we used a selective culture medium. Using semi-selective culture medium, which supports the growth of bifids and lactobacilli, only *B. dentium*, *S. inopicata* and *P. denticolens* were isolated and bifids were present in 13 out of 19 and in 11 out of 15 subjects examined for dental caries and plaque, respectively (26). In this study we have slightly modified a medium previously described for the isolation of non-glucose-fermenting bifids from the cecum of chickens (31). The medium used here was modified with a reduced concentration of mupirocin, from 100 to 5 μg/ml. Mupirocin applied topically as an ointment is effective against gram-positive bacteria especially methicillin-resistant *Staphylococcus aureus*; it is a mixture of several pseudomonic acids but Rada and Petr (31) demonstrated that bifids were less susceptible than other gram-positive bacteria. The source of the peptone in the medium was also changed from a mixture of 'peptone' and soya peptone to a mixture of proteose and soya peptones and raffinose was added to promote the isolation of non-glucose fermenting species (6, 38). These modifications did not significantly affect the utility

of the medium as the growth of non-bifid organisms was sufficiently inhibited and viable counts of type and reference strains were not significantly different (data not shown).

In addition to the usual oral bifids we also isolated *Scardovia* genomosp. C1, reported previously from carious dentine (27), and a number of non-oral bifids including *B. breve*, *B. subtile*, and *B. longum*. In earlier reports (8, 17, 19), non-oral bifids including '*B. breve*-like' organisms, *B. longum*, *B. adolescentis*, and *B. urinalis* have been isolated from the oral environment. These data suggest that non-oral bifids may transiently colonize the oral cavity, although we isolated *B. subtile* from lesions in the same individual (data not shown) indicating colonization of carious root lesions by non-oral bifids, and may suggest that non-oral bifids, including bifids used as probiotics, may colonize carious teeth.

The proportion of bifids in the clinical samples was significantly related to the caries status of the root lesions as we have previously demonstrated for the caries-associated microorganisms, mutans streptococci, lactobacilli, and yeasts (5), indicating that the acidic environment of the lesions provided a suitable habitat for the proliferation of these aciduric microorganisms (7). The numbers of these organisms in the mouth perhaps estimated using saliva-derived counts might be an additional marker for an oral caries-promoting environment in which aciduric bacteria proliferate. No doubt these microorganisms are involved in the progression of caries lesions, a consequence of their ability to produce acid and survive in the acidic environment of the infected dentine but, as so many previous studies have indicated, lesion progression is not dependent upon the presence of an individual organism (4, 25). Dental caries is a biofilm-mediated disease and the range of microbial populations able to progress the demineralization of dentine may be unknowable given the diversity of the cultivable and as yet uncultivable organisms detected in the oral biofilm associated with both sound tooth surfaces and within infected dentine. Furthermore, it seems premature on the basis of the small numbers of lesions from the enamel surfaces of the deciduous or permanent dentitions and root caries lesions that have been investigated and the apparent bias in the cloning data to suggest that there is a characteristic microbiota associated with each type of carious lesion.

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